CHARACTERISTICS OF A FACTOR PROTECTING THE VIABILITY OF LYOPHILIZED *BRUCELLA ABORTUS* CELLS[1]

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The ability of some microorganisms to survive a stress treatment such as freeze drying has been shown to improve when aging the cells is done before drying. Hutton and Shirey (1951) described the beneficial effects of adding filtrates from aged cultures to cell paste of *Brucella abortus* before freeze drying. Record and Taylor (1953) showed that an exudate of *Escherichia coli* offered protection to *Escherichia coli* and to other organisms as well. These investigators have suggested that this biological activity is due to a substance from bacteria and that it is effective in relatively small concentrations. We have studied the action of this viability protective factor (VPF) and have separated it from other constituents of the bacterial exudate. Also it has been partially characterized. The work reported here concerns the extraction of VPF from *Brucella abortus* and the measurement of its activity upon the survival of this organism during freeze drying.

MATERIALS AND METHODS

The culture employed was *Brucella abortus* BAI strain A-19 grown in a tryptose-cerelose broth at 32 C. This medium had the following composition in g/L: tryptose (Difco), 20.0; cerelose, 10.0; NaCl, 5.0; and 10 ml of a Seitz filtered solution containing 25 mg of thiamin hydrochloride (per L) which was added aseptically after the other ingredients had been autoclaved. Two per cent agar was added when a solid medium was desired.

In order to obtain cell paste for the production of VPF, 1 ml of a 48 hr shake culture was inoculated into 100 ml of sterile broth contained in a 250 ml Erlenmeyer flask and incubated for 4 days at 35 C. As many as forty such cultures were accommodated upon a rotary shaker (Brunswick Scientific Company) operated at 250 rpm.

VPF production. The cells from 4 L of media were separated using a Westphalia centrifuge and were then washed with 1 L of saline (0.85 per cent NaCl). The washed cells were then diluted to a concentration of 10^9 per ml with a tryptose diluent containing 0.1 per cent tryptose (Difco) and 0.5 per cent NaCl. After incubating this slurry for 5 days at 35 C the cells were removed by centrifugation. The cell free supernatant contains an active principle, the existence of which was first indicated in the report of Hutton and Shirey (1951), and which we have termed the viability protective factor (VPF).

Assay of VPF activity. A cell paste obtained from 48 hr surface growth on agar plates was suspended in saline, centrifuged and then resuspended in tryptose diluent to yield cell counts varying from 1 to 300 $\times$ 10^9 cells per ml. This cell suspension (0.5 ml) was placed into each of as many 1-dr vials as required for the assays. Sterile tryptose (0.5 ml) was added to a number of the vials used in each run to serve as controls. Solutions (0.5 ml portions) undergoing assay were added instead of tryptose diluent to other vials containing the cell suspension.

At least 3 vials of control and 3 of each solution being tested were dried simultaneously in a freeze drying unit. The number of cells surviving in each vial was determined by suspending the dried material in tryptose diluent and plating upon tryptose-cerelose agar. Cell counts of the original paste were made at the same time that the dilutions for drying were prepared. Activity of the VPF is expressed as the per cent of the recovery in test suspensions, with the recovery in the control suspensions equaling 100 per cent.

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Drying technique. The drying chamber consisted of a large pyrex bell jar connected by a hydraulically operated valve to a condensor pot. The chamber contained a platen in contact with circulating coils and exposed to an infrared heater. A dry ice solvent mixture gave a circulating temperature of −45 C. Chamber vacuum was measured by a Hasting Thermocouple Vacuum Gage. Chamber air temperature, platen temperature, and sample temperature were measured by thermocouples connected to a 3-channel Bristol Recorder.

Short cycle (4–5 hr) drying runs were made. Samples were quick frozen on a cold platen under partial vacuum. When chamber pressure was below 100 μ and sample temperature stabilized at −40 to −45 C (15 min after start) warm solvent was supplied to the platen coils, and the heater turned on with current regulated at 55 by a variac rheostat. When the sample temperature reached 40 C, the heater and warm solvent system were turned off. The operation was continued for approximately 2 hr more to make certain the samples were completely dry, and to allow the drier temperature to reach an equilibrium with the room temperature.

Chromatographs. The filter paper disc method of Hack (1953) was generally used in examining for the presence of functional groups in active material. A drop of material dissolved in a 4:1 chloroform-methanol mixture was placed at the center of a 6 cm circle of Whatman no. 1 filter paper. Solvent spread caused the test material to become distributed over a small distance from the point of application. Further distribution and separation was obtained by the dropwise addition of acetone at the center of the disc. Such elution carried the material 5 mm from the origin point, then caused a precipitation which did not migrate further. Dropwise addition of chloroform caused the formation of two bands upon the paper. The inner one was quite faint and was generally considered to consist of inactive impurities. Reagents were sprayed upon these bands in examining for functional groups.

Infrared spectroscopy. Samples were dried at 78 C and 0.001 mm Hg for 20 min, then taken up in chloroform and examined by means of a Perkin-Elmer Model 12C spectrophotometer through the common infrared frequencies.

Esterase activity. Substrates of lecithin and of the active material were prepared by King's (1931) method and were then inoculated with 10 per cent of an active lecithinase filtrate. Flocculation of the clear lecithin suspension within 5 days incubation at 37 C was indicative of enzyme action. The lecithinase was prepared from *Bacillus cereus var. albolactis* (ATCC 7004) by the method described by Wagner (1949) as modified from Colmer (1948). Reference lecithin was prepared from egg yolk by the solvent extraction method of Kampschmidt *et al.* (1953).

RESULTS

VPF separation. The procedure which finally evolved after testing of various procedures and assaying of fractions of the incubated cell supernatant for VPF activity is as follows:

Cell free supernatant or cell paste, incubated
Concentrated by vacuum distillation, dried in vacuum oven at 60 C.

Dried residue
Refluxed 4 hr with 3:1 ethyl alcohol-ethyl ether, inactive residue discarded, extract dried below 60 C.

Extract I
Refluxed 4 hr with petroleum ether, inactive residue discarded, extract dried below 60 C.

Extract II
Extracted at room temperature with repurified ethyl ether, centrifuged, insoluble portion discarded, solvent evaporated under vacuum.

Extract III
Active wax material.

The active wax is cream colored. At times when prepared to this stage it has a red to amber color and if treated with acetone, a whitish, insoluble and active fraction separated. The wax was used in chloroform solution for identification studies. As the fraction became more purified their solubility in water decreased suggesting that the VPF wax had been originally associated with a solubilizing moiety. The VPF wax has also been obtained with the method by the direct extraction of both young and old cells from the incubated slurry.

Activity of VPF. The protective ability in different preparations of the active supernatant of Hutton and Shirey was found to vary over a wide range. Measured VPF activities were sometimes as low as 80 per cent and as high as 194 per cent of the tryptose diluent standard. The mean value from 32 earlier tests comparing survival of cells dried from VPF solutions was 166 per cent with a sigma value of 36. Only

TABLE 1

Relative recovery of Brucella abortus from freeze drying in the presence of substances exhibiting viability, protective factor (VPF) activity

Assayed Material	Activity*
	%
Tryptose supernatant from aged cells	160
Extract I, tryptose supernatant	174
Extract II, tryptose supernatant	182
Extract II, old cells	178
Extract II, young cells	184
Extract II, saline supernatant from aged cells	187

* Activity expressed as per cent of the recovery obtained with tryptose diluent equaling 100 per cent recovery.

TABLE 2

Qualitative tests for functional groups in the viability protective factor of Brucella abortus

Reagent or Test	Functional Group	Reaction
Ninhydrin	α-Amino	−
Biuret	Peptide linkage	−
Molisch	Carbohydrates	−
Benedict	Aldo-, keto-carbohydrates	−
Schiff	Reducing groups	−
Iodine	Unsaturation	+
$KMnO_4 + I_2$ (*a*)	Unsaturation	+
Brom cresol green	Acidity	+
Brom cresol green (*b*)	Fatty acids	+
Phosphomolybdic acid + $SnCl_2$ (*c*)	Choline	+
Lieberman-Burchard	Sterols	−
Molybdic acid (*d*)	PO_4 esters	+
2,4 Dinitrophenyl hydrazine + $HgCl_2$ (*e*)	Acetal lipids	−
Sulphurous Fuchsin + HIO_3 (*e*)	Glycolipids	−
Feigl spot tests	Fe, Cu, Mg, Mn, Zn, Co, Ni	−

(*a*) Huennekens, Hanahan and Uziel (1954); (*b*) Block, LeStrange and Zweig (1952); (*c*) Levine and Chargaff (1951); (*d*) Hanes and Isherwood (1949); (*e*) Hack (1953).

those supernatants having an activity within the one sigma limit (130 per cent or more) were purified for chemical study. Table 1 shows typical values of the VPF activity of various fractions and extracts which are identified in the method shown above. Corrections have been made for differences in concentration of the solutions. A VPF wax, for example, was dissolved in sufficient tryptose diluent before assay to equal the volume of the original supernatant from which it was prepared. Note that highly active materials also were obtained by direct chemical extraction of cells.

Establishment of chemical moieties. VPF wax was examined by a number of tests for particular functional groups. The results are summarized in table 2 and indicate that the material is an organic phosphate ester containing choline and unsaturated fatty acids. An inactive fraction of some preparations which was soluble in acetone was found to be mostly unsaturated fatty acid. It is possible that this was attached to the active portion earlier in the extraction process and may contribute to the greater solubility of the original substance.

An inactive filtrate was found to contain trimethyl amine. This results from the breakdown of choline. Its presence in the inactive preparation is further confirmation of the necessity of choline in active VPF and may explain the inactivity of this particular preparation.

The only common choline containing phosphoesters are the cephalins, lecithins, and sphingomyelins. Cephalins would be ruled out for VPF wax due to absence of a positive ninhydrin test. Sphingosine, a portion of sphingomyelins, is also ninhydrin positive and the VPF wax is not. The sphingomyelins do not contain glycerol but burning of VPF wax gives a distinct acrolein odor. Thus, strong evidence suggested that the VPF of *Brucella abortus* is a lecithin containing unsaturated fatty acids.

Infrared confirmation. Figure 1 shows the infrared spectrum of the active wax between 1400 and 1900 cm^{-1}. The finger print region of

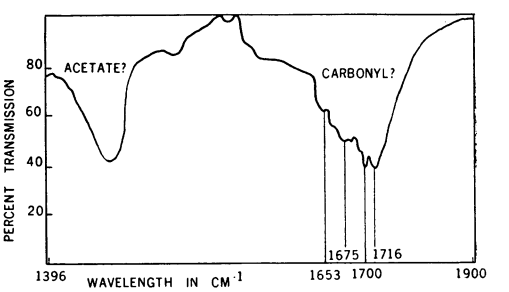

Figure 1. Infrared spectrum of viability protective factor wax.

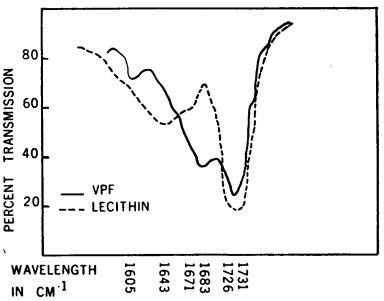

Figure 2. Comparison of the absorption spectra of viability protective factor and egg lecithin (solvent extract and purified) in the "carbonyl" range.

TABLE 3

Effect of egg lecithin and viability protective factor (VPF) upon the recovery of lyophilized Brucella abortus

Added	Protective Activity*
	%
Lecithin 0.25 per cent	50
Lecithin 0.125 per cent	175
Lecithin 0.062 per cent	181
VPF Extract III 2 ×	164
VPF Extract III 1 ×	186
VPF Extract III 0.5 ×	123

* Activity expressed as per cent of the recovery obtained with tryptose diluent equaling 100 per cent recovery.

800 to 1200 cm^{-1} did not yield absorption peaks of importance. This suggests that VPF is highly paraffinic in character. The peaks between 1653 and 1716 cm^{-1} could result from unsaturated bonds conjugated with carbonyl groups. Myristic and palmitic acids have peaks at 1710 and 1716 cm^{-1}.

Figure 2 compares the infrared absorption of VPF substance and lecithin extract from egg yolk in the most absorptive ranges. Considerable similarity exists between the two curves though the VPF peaks are not as complex. The strongly defined peaks at 1726 and 1731 cm^{-1} seem to indicate the presence of an ester carbonyl linkage associated with well purified fats, cholesterols and phospholipids.

The extra absorption of VPF at 1605 cm^{-1} is a possible indication of a double bond such as those occurring in unsaturated fatty acids. VPF is therefore concluded to be more unsaturated than egg lecithin.

Enzymatic confirmations. Further evidence that VPF is a lecithin substance was gained by showing that it serves as a substrate for the enzyme lecithinase. Lecithinase extracted from *B. cereus* reacted similarly upon VPF materials and known lecithin extracted from egg yolk. A characteristic flocculation occurred with each substrate again indicating that VPF is lecithin-like in nature.

Influence of egg lecithin on bacterial recovery. If other lecithins exhibited protective properties similar to VPF this would further substantiate the chemical nature of VPF wax. Table 3 shows a comparison of the survival of *B. abortus* upon the additions of lecithin from egg yolk and the active VPF wax obtained from *B. abortus*, at various dilutions. The similarity of the protective activity is evident.

DISCUSSION

The presence of a factor in the supernatant of incubated *B. abortus* cultures capable of protecting the viability of lyophilized cells has been confirmed by the experiments described. The active substance has been obtained by solvent extraction from fresh cells, from the supernatant of saline or tryptose suspensions of cultures, and from the cell residues of the saline and tryptose suspensions. The initial unpure extract is more soluble in water than it is after purification. Hutton and Shirey (1951) have shown that the factor is reasonably heat stable and can pass through a cellophane membrane. This suggests that in the cell it may be combined loosely with a substance (such as a carbohydrate or fatty acid) which assists in its dispersion or solubility.

The activity of the factor is quite variable in different preparations. In view of its identification as a lecithin this would be expected from differences in oxidation. In fact, lecithin may well exert its protection because of its antioxidant properties. Naylor and Smith (1946) have established the value of ascorbic acid and other antioxidants in increasing the number of *Serratia marcescens* cells surviving freeze drying. Also, Kampschmidt, Mayer and Herman (1953) have shown increased resistance of bovine spermatozoa to cold shock when egg yolk is included in the freezing menstrum.

The identification of VPF as a lecithin is based on chemical tests on the active waxy material that has shown that it contains unsaturated bonds, fatty acids, an organic phosphate ester, glycerol and choline. Its infrared absorption proved similar to that of other lecithins. It also was found to serve as a substrate for bacterial lecithinase and its action was shown to parallel that of egg lecithin in the protection of *B. abortus* against the detrimental effects of freeze drying. Upon this evidence it is concluded that the VPF of *B. abortus* is a lecithin containing at least one unsaturated fatty acid in its molecule. The position of the choline phosphate upon the glyceride and the size and type of fatty acids are as yet undetermined.

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SUMMARY

A viability protective factor that enhances the survival of *Brucella abortus* during freeze drying was extracted from the supernatant of aged cultures, and from young and aged cells. The factor was purified to a waxy material by solvent extraction. Chemical and enzymatic reactions, as well as spectroscopical characteristics, have led to the assignment of a lecithin structure to the active compound.

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